STUDIES ON CHROMOGENIC BACTERIA. I.

NOTES ON THE PIGMENT OF BACILLUS POLYCHROMOGENES.

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(WITH SIXTEEN FIGURES)

THE *Bacillus polychromogenes* was first isolated from a well water of Nancy by Macé[1] in 1894, and since its first discovery he has met with this same organism on five different occasions in well and conduit waters of that city. Two years after its discovery it was again isolated from the same well, and was found to possess the same characters as in the previous case. The organism was then described by one of us under the name of *Bacille polychrome*.[2]

These six colonies, found at different times, have varied neither in the original colonies nor on subsequent cultivation; varieties are, therefore, still unknown. Neither has it been possible to obtain variation by culture methods, for although one of the original colonies has been grown in the laboratory since 1894, part of the time in America, no change has been observed. It has also been impossible to obtain a non-chromogenic variety in spite of all attempts. It seems more than probable that this beautiful species will be met with by other investigators, and therefore, although the present article has to deal with the pigment, a few words regarding the characteristic features of the bacillus may not be out of place.

The *B. polychromogenes* was so named because of its peculiar power of giving a multiplicity of colors on ordinary culture media. On such media the organism produces at times blue, at

[1] MACÉ, E.: Traité pratique de Bactériologie. Ed. 3. 849–852. 1897. Atlas de Bactériologie, *pl 29*.

[2] THIRY, G.: Sur une bacterie produisant plusieures couleurs. C. R. de la Société de Biologie, 7 Nov. 1896.

Contribution a l'étude du polychromisme bactérien. Archives d. physiol. V. 9: 284–289. 1897.

others red, green, violet, purple, or yellow. These colors, as will be shown, can generally be controlled, thus permitting one to obtain at least part of the colors with certainty. This interesting fact has already been pointed out by the authors.[3] In addition to the pigment described below, colored insoluble microscopic crystals are also generally found on solid media. These crystalline aggregates are made up of irregular radiating clusters of fine needles of a deep blue color, and are probably not due to a crystallization of the pigment, but to crystals of some other substance stained by it. The composition of these crystals will be discussed in a future communication.

The organism liquefies gelatin, solidified blood serum, albumin (white of egg), fibrin, etc. In peptone solutions no indol is produced, neither does the bacillus produce gas in fermentation tubes filled with glucose or lactose peptone solutions at room temperature nor at incubator temperature.

The bacilli themselves are generally colorless, rarely they appear red or blue; in some of these latter cases the organism is uniformly stained, at others times only minute intra-bacillary granules are colored. The bacillus is polymorphic; not only does its form vary according to the nutritive media employed, but often there is great variation in form observed in different portions of the same medium. It has not been possible thus far to obtain with any degree of certainty a constant form on any culture medium, not even when employing one of definite composition (*i. e.*, a medium in which the source of nitrogen is not peptone etc., but a chemical compound of known composition such as asparagin or other bodies). In general the organism assumes the form of a short rod rounded at the ends, at other times it is spherical; again, long curved giant forms with swollen ends are seen. The bacilli are sometimes isolated, sometimes grouped (diplo-bacilli, chains). Coccus forms are likewise to be found in staphylo- and diplo- forms, or as tetrads and chains of eight cells.

[3] CHAMOT, E. et THIRY, G.: Bacille polychrome. Cultures et Spectre du Pigment. Communication à la Réunion Biologique de Nancy, Feb. 1898.

Usually the organism is motile, but the motion is always slow. Motility is to be seen in colored as well as in colorless individuals. It stains well by the ordinary methods and retains the stains by the method of Gram and that of Claudius. Greater morphological details would take us beyond the scope of the present note, namely, an account of some of the results of the study of the pigment produced by this bacillus on potatoes and on gelatin.

Growth on potatoes and the pigment formed.—On a medium as variable in composition as potatoes of different varieties and different ages of growth, it might be expected that there would be considerable variation in the colors of the pigment and nature of the growth of a chromogenic organism. The *B. polychromogenes* shows the effect of such changes in a most marked manner. Potatoes upon which this organism grows are colored variously yellow, greenish, red, violet, blue; the last color predominating but not constant. It was soon found, however, that a beautiful deep blue could be obtained, almost without fail, if the potatoes were first soaked in a dilute solution of sodium hydroxid (0.25 per cent. to 0.50 per cent.) containing a little calcium phosphate for twenty-four hours or less, depending upon the thickness of the pieces. Since this medium has served as the basis for the isolation of the pigment, and is being constantly employed by us in the study of the pigments of other chromogenes, it may not be out of place to describe our methods of preparation.

As large tubers as possible are chosen, such as are known to become mealy and porous on boiling. They are well washed in cold water, using a brush to aid in cleansing, and are dropped into boiling water with the skins on, and boiled till just cooked through. The water is then poured off, the potatoes allowed to cool somewhat, pealed, cut into slices 1 to 2^{cm} thick, and dropped into a dilute solution of sodium hydroxid, where they remain about eighteen hours. The supernatant liquid is then poured off, the slices drained and transferred to glass boxes 100^{mm} in diameter and 49 to 50^{mm} deep, with loosely fitting covers (in other words deep Petri dishes), a little water is added, and the

medium sterilized three days in succession in streaming steam.

When an active culture of *B. polychromogenes* is inoculated upon such a piece of potato, a deep blue very soluble pigment is produced, which diffuses slowly through the whole mass of the medium, coloring the latter an intense indigo blue. In from ten to fifteen days the coloring matter has penetrated the entire mass, and has colored it uniformly and so intensely that the slice of potato appears almost black. The more porous the potato, the more thoroughly and uniformly is it colored, since the pigment is reduced in the absence of air. For some time the color remains of the same intensity, then it becomes violet or purple, and then, owing either to the organism being no longer able to produce pigment to replace that being reduced by the various reducing substances present in the potato, or owing to the production of reducing agents by the culture itself, the color begins to fade. Decolorization proceeds more and more rapidly, the color at the surface of the medium exposed to the air being the last to disappear. The culture medium finally assumes a dirty brown color. Generally, cutting up the potato into thin slices and exposing them to the air leads to the production of a blue again by oxidation, providing the culture is not too old.

The blue pigment is very soluble in water, quite soluble in *dilute* alcohol, insoluble in strong alcohol, in ether, chloroform, benzine, etc. Water, therefore, is the best solvent; but unfortunately extraction with water removes such an amount of reducing substances that it was found necessary to employ dilute alcohol, the strength of the latter varying with the moisture present in the culture to be extracted.

In order to extract the pigment, the piece of culture medium is cut into thin slices and exposed to the air for a short time in order that as much of the blue pigment as possible shall be formed. Dilute alcohol is then poured over the material and allowed to act for some hours, the blue alcoholic solution is poured off, and a fresh addition of the solvent is made. This is repeated as long as any coloring matter can be removed. The

alcoholic solution of the pigment is then filtered through a carefully cleaned bacterial filter (Chamberland, Kitasato, d'Arsonval, or others). The first portions of the filtrate are rejected, owing to the fact that a chemical action takes place at first, doubtless owing to air present in the pores of the tubes; the result is the production of a beautiful purple-red liquid. After some 50^{cc}, more or less, have passed, the filtrate passes unchanged in color. It is then evaporated to a syrupy consistence at 50° to 60° C.; a higher temperature leads to reduction and decomposition of the pigment at this stage, owing to the presence of large amounts of sugars extracted from the potatoes. The thick deep-blue liquor is precipitated with strong alcohol (about 98 per cent.), the supernatant liquid poured off, and the precipitated pigment dissolved in a very small amount of distilled water and again reprecipitated with alcohol. This process is repeated as long as the alcohol seems to extract anything from the pigment. At this point the pigment is precipitated in such a finely divided condition that it refuses to settle completely, and cannot be retained on filter paper; a very small bacterial filter is therefore employed for the separations. The material is then carefully removed from the filter tube and dried. This dry amorphous powder has a grayish-blue color, and is completely soluble in water to a beautiful pure-blue color. Although we have reason to believe that it is still impure, the amount of impurity is doubtless so small that the pigment thus separated can be used as the basis of comparison, and for the reactions given in this paper. It cannot be made to crystallize, and is insoluble in all ordinary solvents, such as ether, petroleum ether, benzene, chloroform, amyl alcohol, etc.

If the blue aqueous solution is treated with a trace of acid, the blue is changed to a violet, a trifle more acid leads to the production of a beautiful purple (royal purple). An excess of acid gives rise to a red with more or less of a purple tint. So sensitive is the compound to acids that carbon dioxid causes a change of color. It was at first thought that when organic acids were employed, a color change resulted which was different

from that produced by inorganic acids. Later experiments seem to indicate that this is more probably due to a difference of intensity of action.

Ammonium hydroxid causes a change similar to that produced by acids, but the red color in this case is of a different tint from that obtained with the latter. The addition of acids to the purple-red ammoniacal solution restores the blue color, but if the acids are added in great excess the red tint of acid solutions results. It is worthy of note that a decided excess is necessary.

Fixed alkalies (potassium, sodium, barium hydroxids), in small amount, first produce a violet tint; if a little more of the reagent is added a pure blue results, but the color is somewhat paler than that of the original solution. When added in excess, the fixed alkalies give rise to a grass green solution. When the pigment has not been carefully purified, or when filtrates directly from a culture are employed, the change to green is much more rapid, and the amount of alkali required for its production is less. If the pigment is quite free from foreign bodies the green is rather persistent, but when impure rapidly fades away, leaving a yellowish liquid. The process of decolorization begins at the bottom and gradually extends upwards until the surface is reached; here, being in contact with the oxygen of the air, the color persists. If the yellowish alkaline liquid be shaken with air it immediately turns green, then blue; and if the agitation be continued there results a blue solution of almost the same intensity as the original. Allowed to stand undisturbed a reverse change is observed, namely, rapid decolorization passing through a green. The blue can be restored even after several days by shaking with air. The addition of alcohol to the yellowish solution produces a dirty yellow precipitate which turns blue the instant it comes in contact with oxygen. This phenomenon explains why it is that porous potatoes yield most pigment, and why cutting the colored medium into thin slices and exposing it to air before extracting, gives a larger quantity of the coloring matter; for it seems to be obvious that we have to do here with a case of oxidation.

Fixed alkalies added to the red solution resulting from the action of acids on the blue first restore the blue color, then, if in excess, produce a green, which in turn disappears as has just been described; and in like manner shaking with air or addition of hydrogen peroxid restores the blue. From neither acid nor from alkaline solution will solvents such as petroleum ether, ether, chloroform, benzene, amyl alcohol, etc., extract any coloring matter.

As to the important question whether the organism produces the blue pigment or a compound which turns blue in air, the writers do not yet feel justified in advancing an opinion.

When the clear blue solution (obtained by dissolving in water the coloring matter isolated from potatoes by the method described above) is placed before the spectroscope, a fairly well defined absorption band is seen in the neighborhood of the D line. The maximum intensity of this absorption band has approximately a wave-length of $\lambda = 594$, in the case of the purest pigment thus far obtained; its width and intensity varies, naturally, with the concentration and thickness of layer of the solution examined. There is also a slight darkening of the spectrum in the red, and a similar cutting off in the blue extending to the far violet. In the red this absorption seems to begin somewhere from $\lambda = 680$ to $\lambda = 690$, but is so gradual that no satisfactory measurements can be made. In the green, blue, and violet the increasing absorption is so gradual that no reliable decision can be made as to just where the absorption begins. *Fig. 1* gives the absorption spectrum of solutions 10^{mm} thick, containing 1^{gm} per liter of the purest pigment obtained; *fig. 2*, same solutions in layers 25^{mm} thick; *fig. 3*, same pigment in solutions of 2^{gm} per liter, examined in layers 10^{mm} thick. In 25^{mm} layers the absorption bands of these last solutions are too intense to permit of their being represented on the same scale as those figured. Blue solutions obtained by mere filtration of water extracts of colored potatoes usually show more marked absorption in the red and violet ends of the spectrum than do solutions of the pigment separated as previously described. *Fig. 16* shows the spectrum

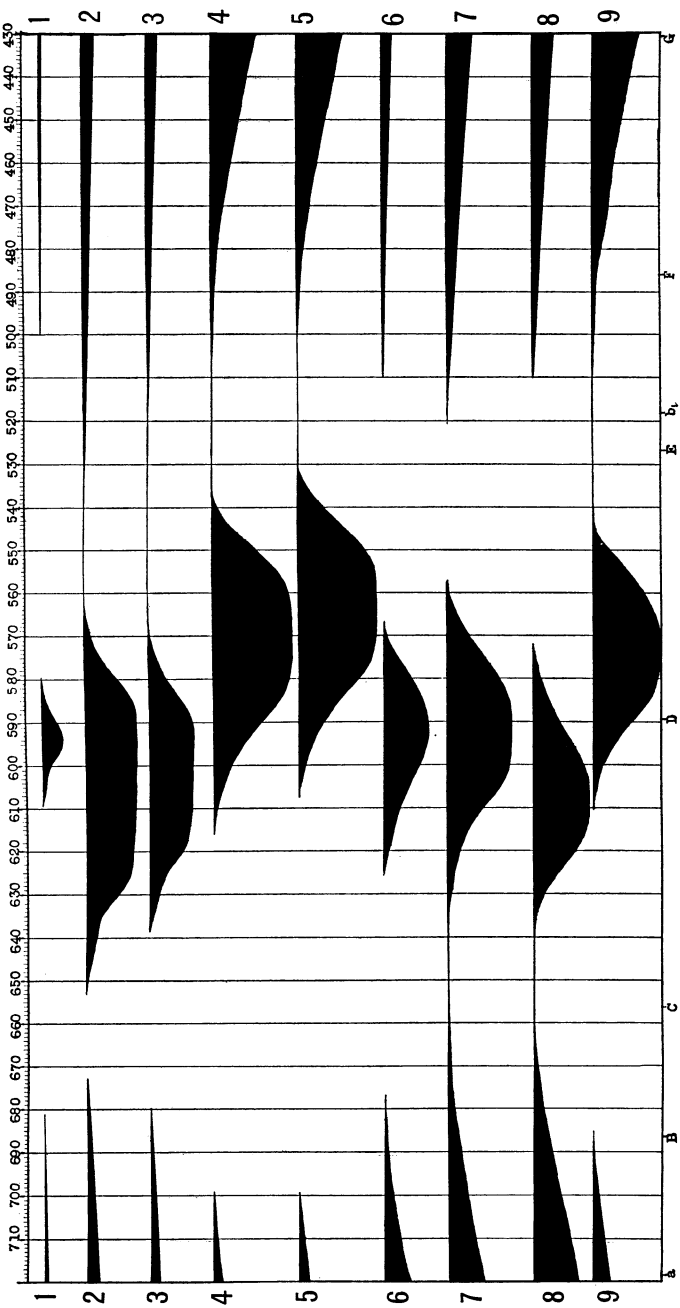

FIG. 1. One gram of pigment per liter in layer 10^{mm} thick.— FIG. 2. One gram of pigment per liter in layer 25^{mm} thick.— FIG. 3. Two grams pigment per liter in layer 10^{mm} thick.— FIG. 4. Two grams per liter, 10^{mm} layer, plus acetic acid.— FIG. 5. Two grams per liter, 10^{mm} layer, plus hydrochloric acid. — FIG. 6. Two grams per liter, 10^{mm} layer, plus ammonium hydroxid. — FIG. 7. Two grams per liter, 25^{mm} layer, plus sodium hydroxid until violet color results. —FIG. 8. Filtered aqueous extract of potato, plus sodium hydroxid 10^{mm} layer. Color intensity about equal to 2 grams pigment per liter. —FIG. 9. Same solution as that used for *figs. 8* after addition of acetic acid. 10^{mm} layer.

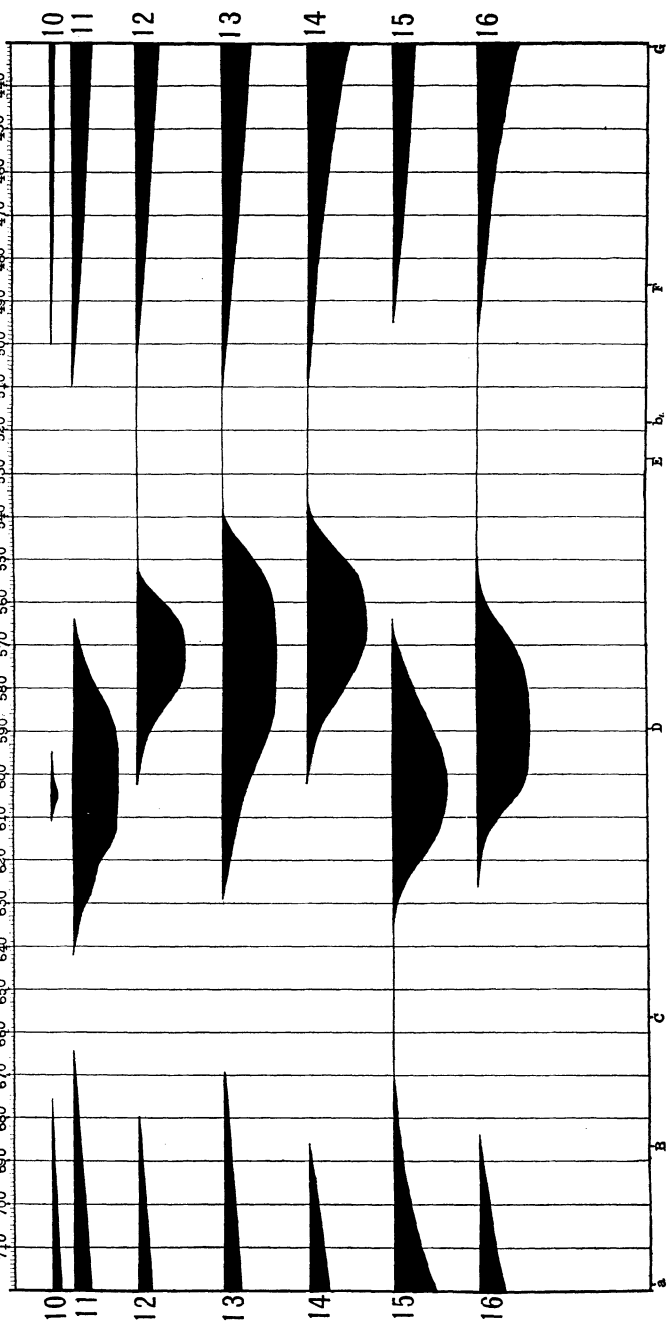

FIG. 10. Two grams pigment per liter, 25^{mm} layer, plus sodium hydroxid in excess.—FIG. 11. Green dichroic solution from gelatin culture three days old.—FIG. 12. Same solution as that used for *fig. 11* after addition of acetic acid. 10^{mm} layer as in *fig. 11*.—FIG. 13. Violet solution from growth on gelatin containing glucose.—FIG. 14. Same solution as that used for *fig. 13* after addition of acetic acid.—FIG. 15. Same solution as *fig. 13* after sodium hydroxid added in excess and mixture shaken with air until blue.—FIG. 16. Filtered aqueous extract from potatoes. 10^{mm} layer. Color intensity about equal to that of 2 grams pigment per liter.

obtained when 10^{mm} layers of such filtrates are examined, after having been diluted to a color intensity approximately equal to 2^{gm} per liter of purified pigment.

The addition of acids to the blue solutions causes an immediate displacement of the main band toward the violet, and increases its intensity at least twofold. The maximum intensity, of the main absorption band, of the now purple-red solutions is about $\lambda=570$. The absorption in the red seems to be diminished, but that in the blue and violet slightly increased. In *fig. 4*, the effect of acetic acid is shown; in *fig.* 5 that of hydrochloric acid. In each case solutions of 2^{gm} of the isolated pigment per liter, in layers 10^{mm} thick, were employed. *Fig. 9* shows the effect of acetic acid on the solutions used in *fig. 16*.

Ammonium hydroxid added in excess to the pure blue solution causes but little displacement of the main band, but greatly reduces its intensity, as also that of the bands in red and violet. This is not the result of dilution alone, however, since the addition of a corresponding volume of water gives no similar reduction in the intensities of the bands. *Fig. 6* shows the action of this reagent in slight excess on solutions of 2^{gm} of the pigment per liter, when examined in layers 10^{mm} in thickness.

Fixed alkalies when added in *very* small amount first produce a violet color. Such solutions yield an absorption spectrum shown in *fig. 7*, in which sodium hydroxid has been added to a 25^{mm} layer of a 2^{gm} per liter solution of pigment. Added in excess until a green results, the absorption band at D is almost completely destroyed, and the other bands about equally reduced in intensity. This effect is shown in *fig. 10*, where solid sodium hydroxid (to avoid dilution) has been added to solutions similar to those used for *fig. 7*. If the alkaline solution be shaken with air until it turns blue, a dark absorption band again appears, but not in the same position as in the original solutions, for it has been displaced toward the red end of the spectrum; its maximum intensity now falls between $\lambda=605$ and $\lambda=610$. This change in position is indicated in *fig. 6*, solutions similar to those used in *fig. 16* being employed.

Growth on gelatin.—Grown on gelatin of standard composition, liquefaction results and a green color is produced which gradually diffuses throughout the medium, the color being most intense near the surface. Generally after a few days a distinct red dichroism (fluorescence?) appears which increases with age.[4]

For the production of the coloring matter in large amount the following method is employed. A 10 per cent. solution of gelatin is made nutrient by adding 1 per cent. peptone (Witte or Chaputeau) and is rendered distinctly alkaline with sodium hydroxid. The medium is then clarified as usual with white of egg. It is then sterilized in the steam sterilizer in the ordinary manner. Gelatin of from 8 to 30 per cent. containing 1 to 5 per cent. peptone gives almost equally good results.

Since the coloring matter is formed only in the presence of an abundance of oxygen, cultures are made in large Fernbach flasks (antitoxin flasks) having a diameter of 20 to 25^{cm} at the bottom, the amount of gelatin added being sufficient to give a depth of about 1^{cm} (about 200^{cc}). The rapid production of as large quantities of pigment as possible being the end in view, the culture flasks thus prepared are inoculated with 1 to 2^{cc} of the liquefied gelatin poured from a very active culture of known purity, and are then placed in a closet protected from the light, where they are kept at a temperature of from 15° to 20°C. If the temperature rises much above 20° the production of pigment diminishes, and ceases if the culture be placed in the incubator. This is true for all culture media; the best results are obtained when the cultures are kept cool.

Fernbach flasks prepared as above begin to show at the end of 18 to 24 hours a decided green color which rapidly increases in intensity. At the end of three days the entire gelatin has assumed an intense, bright, grass-green, owing to the easy solubility of the pigment in water. Day by day the green becomes more intense and slowly darker, liquefaction also begins and with

[4] For diagnostic details the reader is referred to the articles already mentioned.

it a red dichroism makes its appearance; that is to say, the liquefied gelatin when viewed by reflected light is a pure grass-green; when viewed by transmitted light, red. The liquefied gelatin at this time cannot be distinguished in appearance from an alcoholic solution of chlorophyll. Like the latter it loses all its green tint by lamp, or ordinary gaslight (yellow light). That the coloring matter is, nevertheless, not chlorophyll will be seen from its absorption spectrum and from its behavior toward reagents.

The liquefaction of the gelatin starts at the center of the flask, where, owing to the slight convexity of the bottom, the layer of the medium is thinnest. The entire medium is soon completely liquefied, but still retains its intense green color. Gradually, however, the green changes to an olive tint and then fades away, as does also the dichroism, leaving a brownish-yellow turbid liquid.

In order to test the green coloring matter with reagents or to examine it spectroscopically, a perfectly clear solution must be obtained. This is effected by filtering the culture medium through one of the bacterial filters mentioned above. When liquefaction has not yet taken place, sufficient water is added to permit its passage through the filter tubes. When liquefaction is advanced, filtration is at once resorted to. The first runnings are rejected owing to changes in color, the result of chemical action in the pores of the filter.

The green non-dichroic solutions from young cultures give no absorption band in the neighborhood of the D line, or only the trace of one. The addition of a little acid produces a slight dichroism and a faint band appears. Acids added to any of the green dichroic solutions produce a red when in excess. Ammonium hydroxid gives also a somewhat similar color. Fixed alkalies destroy the dichroism and yield a fine clear green soon fading away. Shaking with air restores the color. These green alkaline solutions resemble the green ones obtained from young cultures and from potatoes in that they do not give an absorption band near D. The green pigment is insoluble in alcohol

and in the solvents enumerated above. The absorption spectra will be found represented in *figs. 10–16.*

If to the gelatin, prepared as has been described, a few grams of glucose, lactose, or similar bodies are added, instead of a green, a magnificent blue is obtained, permeating the culture medium completely and uniformly. The blue changes in a few days to a violet, then to a royal purple, and finally, as the culture grows old, the color disappears, the liquefied gelatin acquiring a brownish red color. Both the violet and the purple are dichroic, that is, are red by transmitted light. The liquefaction of the gelatin is considerably retarded by glucose, lactose, etc., as has been observed with many other species of bacteria.

The blue solution obtained by filtering young glucose-gelatin cultures reacts toward reagents in every way like the blue obtained from potatoes, that is, with acids first a violet, then a purple, and at last a red results; ammonia also produces a red; fixed alkalies give a green, soon fading and leaving a brownish-yellow solution which, when shaken with air, becomes first green, then blue. The absorption bands given by these solutions will be found in *figs. 10–16.*

Since it was found that these bands seemed to correspond in character, position, and intensity with those given by similarly colored or treated solutions of the pigment isolated from potatoes, no attempt has yet been made to isolate the coloring matter from gelatin. For the purposes of comparison, the filtered solutions were diluted with water until the colors given with reagents were approximately the same as those obtained with 2^{gm} per liter of the purified pigment.

The spectra of these gelatin solutions need but a few words in explanation. A few of the most important have been given in order that they may be compared with the similarly treated solutions shown by *figs. 1–9.*

Fig. 11 gives the absorption spectrum obtained with strongly dichroic green solutions. Acetic acid added to such solutions causes the change shown by *fig. 12.* *Fig. 13* represents the appearance seen when the violet solutions from cultures on

gelatin containing glucose, lactose, etc., are examined. It will be seen that the main band has suffered displacement toward the right, just as if an acid had acted slightly upon the coloring matter. Addition of acetic acid with the production of a purple-red color furnishes an absorption spectrum like that shown in *fig. 14* (compare this with *figs. 4, 5, 9*). *Fig. 15* is obtained when the violet solutions shown in *fig. 13* are treated with an excess of fixed alkali (in this case sodium hydroxid), and the mixture shaken with air until blue (compare with *fig. 8*).

The absorption curves represented on the plates are drawn to the scale of wave-lengths. The intensities are, of course, arbitrary, and are based upon a value which would permit of representing a spectrum such as that of *fig. 10*. Owing to the difficulty of judging the intensities of most of the solutions examined, particularly after long intervals of time, it is probable that the curves may not be perfectly accurate as to intensity. The positions of the bands, however, are correct within the limit of experimental error. The results given are averages from examinations of many cultures extending over a period of more than two years.

Most of the measurements have been made with a Krüss Universal Spektralapparat. It is, perhaps, needless to add that the usual precautions as to calibration, checking adjustments, etc., were observed.

Growth on agar-agar.—On nutrient agar prepared as usual and rendered slightly alkaline with sodium hydroxid, a more or less bluish-violet color is produced which diffuses through the upper part of the culture, but is reduced where the air cannot penetrate. In this case the reduction of the coloring matter has been proved by experiment to be largely due to the action of the culture medium, and not to products formed by the bacillus. In young cultures the bluish tint is the strongest, in old ones the red tint.

If to the nutrient agar glucose or lactose is added, the blue is very marked, but soon becomes violet, then purple. In the case of media inoculated by streak the change from blue to

violet starts near the inoculation streak and proceeds outward. This seems to give evidence of the formation by the organism of an acid or an acid acting substance. Such a hypothesis is supported by the fact that the red is not produced if calcium carbonate is suspended in the medium before it solidifies, thus furnishing a substance which will unite it with and neutralize any acid as soon as formed.

The comportment of the blue from agar toward reagents is identical with that of the blue from potatoes, and with that from gelatin containing glucose, etc. The absorption spectra of the different colored solutions from agar seem to correspond to those of like color obtained from other media, hence it has not been deemed necessary again to reproduce them.

Generalizations.—It will be noticed that potatoes, glucosed-gelatin, agar (especially if glucosed), etc., all yield a blue becoming violet, then more or less purple. Without taking more space to enumerate experiments, it can be stated that no case has yet been found where a blue has resulted in the absence of a sugar or similarly acting compound.

Agar-agar is probably closely related to the starches, and has been shown by Bauer[5] to contain a compound which is doubtless a sugar and, in all probability, to give rise to the formation of others through the action of acids or alkalies. Hence we have here the necessary substances to produce the blue pigment.

The violet colors on different media seem to be due to the action of an acid or acid acting substance. As the culture grows older there is more of this substance formed, and the violet changes to purple or to a red. This hypothesis is based upon the changes in position and character of the absorption bands; on the comportment of agar cultures containing calcium carbonate; and on the fact that the addition of acids to the blue solutions produce changes in color and in absorption spectra similar to those noticed in the aging of cultures. This acid may

[5] Journ. f. prak. Chem. II. **30**: 367. 1884. See also Lippmann, Chemie der Zuckerarten. Braunschweig. 1895.

possibly be acetic acid, since it has been possible to isolate alcohol and acetic acid from some cultures, yet this is by no means certain, since it is not clear whether these compounds existed preformed, or were the result of changes brought about by the analytical methods employed.

In the light of much experimental evidence extending over a considerable period of time, there seems to be no other hypothesis tenable than that all the different colors produced are simple derivatives of one and the same substance, that is to say, the organism does not produce different pigments on different media, as was first supposed, and as has been stated of many other chromogens.

The reactions and properties of this pigment do not correspond to those given by other investigators who have worked upon coloring matters of similar colors. The greens cannot be chlorophyll, nor can the blues be any one of the many cyanins which have been described. The pigment most closely resembles the coloring matters which have been isolated from lichens and from fungi, yet this resemblance is but slight.

The writers hope in a future communication to be able to announce something definite as to the nature of the pigment, and whether it belongs in reality, as now seems to be the case, to a new class of coloring matters heretofore unreported among the pigments of chromogenic bacteria.

One of the chief difficulties is to obtain sufficient purified pigment, for although its tinctorial power is very great there is but little of the material formed in each culture, and much of this is necessarily lost in the processes of purification. The problem becomes, therefore, in the first place, one of time, and cultivation of the organism on a large scale.

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